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Effects of Sublabeled Rates of Dazomet and Metam-Sodium Applied Under Low-Permeability Films on *Calonectria* Microsclerotia Survival

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Abstract

Infested soil is the primary inoculum source for *Calonectria* spp. for initiating disease in ornamental and forestry crops. The effects of dazomet and metam-sodium on survival of microsclerotia of 28 isolates belonging to 19 *Calonectria* spp. were evaluated in this study under nursery conditions. Two experiments with exotic *Calonectria* spp. in plastic containers in a greenhouse and three trials with endemic species in field plots were performed during different seasons. The containers and plots were artificially infested with *Calonectria* microsclerotia differentiated on carnation leaf tissues. Basamid (dazomet) was applied at 100, 160, 200, 400, and 500 kg/ha, while Divapan (metam-sodium) was applied at 250, 350, 400, 700, and 1,000 liters/ha in both the containers and plots. The fumigants were applied under virtually and totally impermeable films. Fungal survival was evaluated after 21 days using leaf tissues collected from treated soil and plated on potato dextrose agar, and the ability of microsclerotia to cause infection was tested on red clover. The survival of *Calonectria* inocula and microsclerotia decreased with increasing fumigant rates. In the greenhouse trials, where Basamid was applied at 200, 400, and 500 kg/ha and Divapan at 400, 700, and 1,000 liters/ha, no viable microsclerotia were recovered for 14 exotic *Calonectria* spp., whereas viable inocula of *Calonectria hongkongensis*, *C. naviculata*, and *C. sulawesiensis* were retrieved from the fumigated plots. Low rates of Basamid (100 and 160 kg/ha) and Divapan (250 and 350 liters/ha) were less effective at reducing *Calonectria* viability and, for these treatments, the rate of microsclerotia survival was highly variable among the different isolates and species. Furthermore, totally impermeable film significantly enhanced fumigant performance. Relative to endemic *Calonectria* spp., all of the treatments killed microsclerotia of *C. polizzii* and *C. pauciramosa* independent from fumigant, rate, and film. This research demonstrated the possibility of reducing the application rates by up to 160 kg/ha for Basamid and 400 liters/ha for Divapan under low-permeability films (virtually impermeable film or totally impermeable film) for eradicating or reducing the primary inoculum of *Calonectria* spp. in soil.

Calonectria spp. (also known as *Cylindrocladium*, asexual morph) are widely distributed in tropical and subtropical climates and are pathogens of a broad range of ornamental and forestry crops, especially in nursery settings (Alfenas et al. 2013; Crous 2002; Crous et al. 1991; Lombard et al. 2009, 2010a,b, 2011; Polizzi and Crous 1999; Vitale et al. 2013b). Disease symptoms associated with *Calonectria* spp. include damping-off, blight, leaf spots, crown, and collar and root rot (Crous 2002; Henricot and Culham 2002; Lombard et al. 2010a; Polizzi et al. 2006a,b, 2007a,b, 2009, 2012; Vitale and Polizzi 2008; Vitale et al. 2008, 2009a). The management of infections in nurseries should involve the development of integrated strategies aimed at reducing both the level of the primary inoculum and the rate of infection in contaminated substrate or soil used for cultivation. Chemical control is the most widespread approach for managing Calonectria diseases in nurseries (Aiello et al. 2013; Cinquerrui et al. 2017; LaMondia 2014, 2015). However, the use of some fungicides such as benzimidazoles and sterol demethylation inhibitors should be limited because they induce a high-selective pressure for resistant isolates (Guarnaccia et al. 2014; Vitale et al. 2009b). Some biological control agents are effective against *Calonectria* infections but their efficacies are variable depending on the application modes and timing, as well as the target species and isolates (Daughtrey and Benson 2005; Harman 2000; Vitale et al. 2012).

A primary inoculum of *Calonectria* spp. consists of microsclerotia which can survive in soil for 15 years or more (Phipps and Beute 1979; Thies and Patton 1970). In nurseries, especially those with replants, the use of infected substrate or soil is the typical source primary *Calonectria* inoculum. Making matters worse, nurseries have recently begun using containers made from recycled and composted organic substrates such as peat, bark, wood fibers, and green waste (Chong 2005; Walters 2009), which can further increase the risk of *Calonectria* spp. infections (Noble and Roberts 2004). Once substrates are infested, the eradication of these pathogens can be very difficult. Soil solarization treatments to reduce the potential inoculum have been successfully reported against *Calonectria* spp. (Vitale et al. 2013a). However, the efficacy of soil solarization is strictly dependent on exposure time, the temperature reached in the upper 0- to 30-cm layer of soil, and the specific heat sensitivity of the targeted isolate (Vitale et al. 2013a). Soil fumigation is a more widespread practice for reducing inoculum in intensive cultivation systems; however, only limited studies have addressed *Calonectria* spp. infesting peanut and forest tree nurseries (Crous 2002). According to recent European directives only metam-sodium (MS), metam-potassium, and dazomet (DZ) are authorized for the disinfection of soils/substrates in agriculture. Moreover, Directive 2009/128/EC ("Sustainable Use of Pesticide") of the European Parliament and the Council of the European Union (2009) imposes severe restrictions regarding application methods and rates, including that the fumigants should be applied at reduced rates and under mulching films that decrease gas emissions and environmental damage (European Commission 2012). As a result, our current research has focused on the more advanced multilayer films such as virtually impermeable film (VIF) and totally impermeable film (TIF) that are less permeable to chemicals than the low- and high-density polyethylene films (Fennimore and Ajwa 2011).

A preliminary study recently demonstrated the potential use of labeled rates of DZ and MS applied under VIF for the suppression of *Calonectria* microsclerotia in nurseries of the Mediterranean Basin (Polizzi et al. 2014). However, no data are available on the effects

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of sublabeled fumigant rates and TIF performances against epidemic *Calonectria polizzii* and *C. pauciramosa* and exotic species from forest crops. This information could improve the environmental sustainability of these fumigants.

Thus, we investigated the effects of DZ and MS applied at labeled and sublabeled rates under TIF and VIF films on 17 exotic *Calonectria* spp. in greenhouse trials and the effects of the fumigants applied at sublabel rates on well-established *C. polizzii* and *C. pauciramosa* species in the Mediterranean Basin in open fields under nursery conditions.

Materials and Methods

Pathogen isolates. In total, 28 isolates belonging to 19 *Calonectria* spp. were used in this study, including two species well-established in Italy, *C. polizzii* ITEM 14877 and *C. pauciramosa* ITEM 14884, and 17 exotic species previously identified by a multigene sequence analysis but not currently present in Italy (Table 1). All of the isolates were grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) prior to transfer to carnation leaf agar (CLA) (Fisher et al. 1982) for microsclerotia production.

Effects of DZ and MS on exotic *Calonectria* microsclerotia. Because the exotic *Calonectria* spp. are not present in Italy, experiments to test fungal survival were performed in plastic containers (40 by 60 cm). Two experiments (I and II) were performed in November 2014 and May 2015, respectively, in a greenhouse to determine the effects of DZ and MS on the viability of microsclerotia of 17 *Calonectria* spp. (26 isolates) buried in a soil substrate. The commercial fumigants evaluated were Basamid Granulat (99% DZ, water-dispersible granules; Kanesho Soil Treatment, Brussels, Belgium) and Divapan (51% MS, suspension concentrate; Taminco Italia s.r.l., Milano, Italia).

Microsclerotia were produced on CLA after 2 weeks of incubation at 25 ± 1°C, and two carnation leaf segments (6 cm long) colonized by pathogen microsclerotia were removed from Petri dishes and placed in a nylon mesh bag. Three bags for each isolate at each fumigant rate were buried (15 cm in depth) in plastic containers filled with a loamy-sand substrate.

Basamid (DZ) was applied at rates of 100, 160, 200, 400, and 500 kg/ha and mixed uniformly into the soil before the bags were

Table 1. Fungal isolates collected from ornamental and forest crops used in this study

Isolate code[y]	Location	Host	Other collections (nr.)[z]	Species	Species complex	References
LPF130	Suzano-BA	*Eucalyptus* sp.	…	*Calonectria brachiatica*	*C. brassicae*	Lombard et al. 2009
LPF195	Suzano-BA	Native forest	…	*C. brachiatica*	*C. brassicae*	Lombard et al. 2009
LPF034	Jari	*Eucalyptus* sp.	…	*C. brassicae*	*C. brassicae*	Lombard et al. 2009
LPF290	Suzano-BA	*Eucalyptus* sp.	…	*C. brassicae*	*C. brassicae*	Lombard et al. 2009
LPF452	Jari	*Eucalyptus* sp.	…	*C. ecuadoriae*	*C. brassicae*	Crous et al. 2006
LPF300	Jari	*Eucalyptus* sp.	…	*C. orientalis*	*C. brassicae*	Lombard et al. 2010c
LPF301	Jari	*Eucalyptus* sp.	…	*C. orientalis*	*C. brassicae*	Lombard et al. 2010c
LPF031	Jari	*Eucalyptus* sp.	…	*C. pini*	*C. brassicae*	Lombard et al. 2010c
LPF253	Amcel	*Eucalyptus* sp.	…	*C. pini*	*C. brassicae*	Lombard et al. 2010c
LPF388	Suzano-MA	*Eucalyptus* sp.	…	*C. brasiliensis*	*C. cylindrospora*	Lombard et al. 2010c
LPF244	Viçosa-MG	*Piptadenia gonoacantha*	CBS133608	*C. hodgesii*	*C. cylindrospora*	Alfenas et al. 2013
LPF245	Viçosa-MG	*Anadenanthera peregrina*	CBS133609	*C. hodgesii*	*C. cylindrospora*	Alfenas et al. 2013
LPF389	Suzano-MA	*Eucalyptus* sp.	…	*C. sulawesiensis*	*C. cylindrospora*	Lombard et al. 2010c
LPF007	Jari	*Eucalyptus* sp.	CPC18775	*C. variabilis*	*C. cylindrospora*	Crous et al. 1993a
LPF220	Amcel	*Eucalyptus* sp.	…	*C. variabilis*	*C. cylindrospora*	Crous et al. 1993a
LPF174	Maranhão	*Eucalyptus* sp.	…	*C. ovata*	*C. pteridis*	Victor et al. 1997
LPF257	Amcel	*Eucalyptus* sp.	…	*C. ovata*	*C. pteridis*	Victor et al. 1997
LPF002	Jari	*Eucalyptus* sp.	CPC18771	*C. pteridis*	*C. pteridis*	Crous et al. 1993b
LPF004	Jari	*Eucalyptus* sp.	CPC18773	*C. pteridis*	*C. pteridis*	Crous et al. 1993b
LPF075	Araponga-MG	Native forest	CPC18745	*C. colombiana*	*C. candelabra*	Lombard et al. 2010c
DISTEF173	Lamezia-Catanzaro	*Callistemon citrinus*	ITEM14884	*C. pauciramosa*	*C. candelabra*	Schoch et al. 1999
DISTEF87	San Marco-ME	*Acacia retinodes*	ITEM14877	*C. polizzii*	*C. candelabra*	Lombard et al. 2010b
LPF066	Araponga-MG	Native forest	CPC18736	*C. spathulata*	*C. candelabra*	Crous et al. 1994
LPF061	Suzano-BA	*Eucalyptus* sp.	…	*C. pseudoscoparia*	*C. candelabra*	Lombard et al. 2010c
LPF212	Alagoas	*Eucalyptus* sp.	…	*C. pseudoscoparia*	*C. candelabra*	Lombard et al. 2010c
LPF219	Suzano-BA	Native forest	…	*C. zuluensis*	*C. candelabra*	Lombard et al. 2010b
LPF311	Jari	*Eucalyptus* sp.	…	*C. naviculata*	Sphaero-naviculate group	Crous et al. 1994
LPF111	Unknown	Unknown	…	*C. hongkongensis*	Sphaero-naviculate group	Crous et al. 2004

[y] LPF = Laboratório de Patologia Florestal, Universidade Federal de Viçosa, Viçosa, Brazil and DISTEF = Dipartimento diAgricoltura, Alimentazione e Ambiente, Catania, Italy (Vitale et al. 2012).

[z] CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands; CPC = P. W. Crous working collection housed at CBS; and ITEM = numbers as reported by Vitale et al. (2013a).

buried, whereas Divapan (MS) was applied as soil drench with a hand sprinkler at rates of 250, 350, 400, 700, and 1,000 liters/ha. Following fumigation, the containers were brought to water field capacity, covered, and hermetically closed with VIF (Ecobrom; AgriPlast s.r.l., Vittoria, Ragusa, Italy) or TIF (Eval, Kuraray, Houston, TX) by fixing their edges at the container margins with adhesive tape. Subsequently, containers were placed in a greenhouse. The same number of bags and infected carnation leaf pieces buried in untreated substrate in plastic containers served as the controls. Three replicates (three plastic containers) were used for each isolate, rate, and tested film and arranged in a randomized complete block design applied to each fumigant separately. The nylon bags were sampled 21 days after treatment. Nine leaf pieces, obtained by cutting each leaf segment, were washed with sterile distilled water (SDW), placed onto PDA plates, and maintained for 1 week at 25 ± 1°C. The percentage of leaf pieces from which pathogen colonies developed was used to determine the survival of single *Calonectria* isolates after recovery from soil.

Effects of reduced fumigant rates on the survival of *C. polizzii* and *C. pauciramosa* microsclerotia. Experiments III, IV, and V were performed in November 2013 and May and July 2014, respectively, in open fields to determine the effects of sublabeled rates of DZ and MS on the viability of microsclerotia of *C. polizzii* and *C. pauciramosa* isolates. A 40-cm layer of soil was placed on a cement bed. The experimental plots, each 2.5 by 5.0 m, were arranged in a randomized complete block design applied to each of both species separately, with three replicates for fumigant rate and tested film. Microsclerotia were produced on CLA as described above, and two carnation leaf segments (6 cm long) colonized by pathogen microsclerotia were removed from Petri dishes and placed in a nylon mesh bag. Three bags for each isolate at each fumigant rate were buried (15 cm in depth) in the soil and filled with loamy-sand soil. Basamid (DZ) was applied at 100, 160, and 200 kg/ha and mixed uniformly into the soil before the bags were buried, whereas Divapan (MS) was applied as a soil drench by a hand sprinkler at 250, 350, and 400 liters/ha. After fumigation, the soil in each plot was irrigated to field capacity and covered with sheets of VIF or TIF. The film sheets were laid on the soil surface and their edges were buried 20 cm deep. The same number of carnation leaf pieces buried in the untreated soil plot served as the control. At 21 days after treatment, nine leaf pieces, obtained by cutting each leaf segment, were washed with SDW, placed onto PDA plates, and maintained for 1 week at 25 ± 1°C. The percentage of leaf pieces from which pathogen colonies developed was used to determine the survival of *Calonectria* spp. after recovery from the soil.

Evaluation of the viability of *C. polizzii* and *C. pauciramosa* microsclerotia on red clover following fumigation treatments. After a 21-day exposure time to fumigation, carnation leaf samples were retrieved from both untreated and fumigated plots, and microsclerotia viability was assayed on red clover (*Trifolium pratense* L.) seedlings according to a method reported in recent articles (Vitale et al. 2012; Waipara et al. 1996). Additional bags containing two infected carnation leaves were previously buried in each plot (replicate) for all treatments. The retrieved carnation leaf samples were cut into smaller pieces (18 pieces per replicate) and mixed with peat substrate in an aluminum tray. The aluminum trays were placed in a growth chamber and brought to water field capacity before seeding the red clover (three replicate aluminum trays, each containing up to 70 seeds). The percentage of red clover seedlings showing crown and root rot symptoms of the total number of examined seedlings was recorded 8 to 10 days after clover seeding.

Statistical analyses. Data from independent experiments (I to V) were analyzed separately by using the Statistica package software (version 10, Analytical Software for Windows; Statsoft Inc., Tulsa, OK). An analysis of variance was performed for each fumigant separately to evaluate the viability among *Calonectria* isolates exposed to single rates and to compare the performances of VIF and TIF in greenhouse experiments I and II (Table 2). For both experiments, results were analyzed in independent experiments based on year (season) and tested fumigant. Subsequently, analyses were conducted by calculating *F* values and significances (*P*) associated with the experimental factors (isolate, rate, and film) and whether there were significant interactions among these factors within each independent

Table 2. Analysis of variance effects of involved factors and their relative interactions on *Calonectria* spp. survival (%) in plastic containers from independent experiments

Year (season)	Fumigant	Source of variation	DF[y]	MS[y]	*F* value	Significance[z]
2014 (autumn)	Dazomet	Isolate	25	1,376	32.8	***
	…	Rate	4	4,859	115.9	***
	…	Film	1	375	8.9	**
	…	Isolate × rate	100	852	20.3	***
	…	Isolate × film	25	305	7.3	***
	…	Isolate × rate × film	100	211	5.0	***
	Metam-sodium	Isolate	25	7,294.9	25.6	***
	…	Rate	4	263,479.8	924.7	***
	…	Film	1	11,708.0	41.1	***
	…	Isolate × rate	100	2,411.6	8.5	***
	…	Isolate × film	25	1,511.7	5.3	***
	…	Isolate × rate × film	100	1,178.3	4.1	***
2015 (spring)	Dazomet	Isolate	25	5,860	33.7	***
	…	Rate	4	52,853	304.4	***
	…	Film	1	7,729	44.5	***
	…	Isolate × rate	100	2,306	13.3	***
	…	Isolate × film	25	551	3.2	***
	…	Isolate × rate × film	100	468	2.7	***
	Metam-sodium	Isolate	25	1,920.0	11.9	***
	…	Rate	4	97,912.5	608.2	***
	…	Film	1	19,560.9	121.5	***
	…	Isolate × rate	100	1,159.3	7.2	***
	…	Isolate × film	25	1,003.0	6.2	***
	…	Isolate × rate × film	100	637.2	3.9	***

[y] DF = degrees of freedom and MS = mean square.

[z] Asterisks ** and *** = significant at 0.001 < *P* < 0.01 and *P* < 0.001, respectively.

Table 3. Comparison of the effects of dazomet (Basamid) on microsclerotia viability reductions (%) when applied to different *Calonectria* spp. in plastic containers under virtually impermeable film (VIF) or totally impermeable film (TIF) in autumn 2014 (experiment I)

	Reductions (%) per rate^z					
	100 kg/ha		160 kg/ha		200 kg/ha	
Isolate	**VIF**	**TIF**	**VIF**	**TIF**	**VIF**	**TIF**
Calonectria brachiatica LPF130	100 a	100 a	100 a	100 a	100 a	100
C. brachiatica LPF195	100 a	100 a	100 a	100 a	100 a	100
C. brassicae LPF034	100 a	100 a	100 a	100 a	100 a	100
C. pini LPF253	100 a	100 a	100 a	100 a	100 a	100
C. orientalis LPF300	100 a	100 a	100 a	100 a	100 a	100
C. orientalis LPF301	100 a	100 a	100 a	100 a	100 a	100
C. ecuadoriae LPF452	100 a	100 a	100 a	100 a	100 a	100
C. pteridis LPF004	100 a	100 a	100 a	100 a	100 a	100
C. ovata LPF174	100 a	100 a	100 a	100 a	100 a	100
C. ovata LPF257	100 a	100 a	100 a	100 a	100 a	100
C. sulawesiensis LPF389	100 a	86.4 b	100 a	100 a	100 a	100
C. pseudoscoparia LPF061	100 a	75.3 c	100 a	100 a	100 a	100
C. hodgesii LPF244	100 a	100 a	100 a	100 a	100 a	100
C. hodgesii LPF245	100 a	100 a	100 a	100 a	100 a	100
C. zuluensis LPF219	100 a	100 a	100 a	100 a	100 a	100
C. colombiana LPF075	100 a	100 a	100 a	100 a	100 a	100
C. spathulata LPF066	98.8 ab	100 a	100 a	100 a	100 a	100
C. variabilis LPF220	96.3 ab	100 a	87.5 a	100 a	100 a	100
C. brasiliensis LPF388	93.8 ab	100 a	100 a	100 a	100 a	100
C. brassicae LPF290	92.6 ab	100 a	100 a	100 a	100 a	100
C. pseudoscoparia LPF212	92.6 ab	85.2 bc	100 a	100 a	100 a	100
C. pini LPF031	91.4 ab	100 a	100 a	100 a	100 a	100
C. variabilis LPF007	85.2 b	100 a	100 a	100 a	100 a	100
C. pteridis LPF002	66.7 c	100 a	100 a	100 a	100 a	100
C. hongkongensis LPF111	33.3 d	53.1 d	98.8 a	74.1 b	100 a	100
C. naviculata LPF311	0 e	56.8 d	66.7 b	100 a	66.7 b	100

^{z} Each value represents the mean of three replicates, each consisting of 54 infected carnation pieces. Recovery values followed by different letters within each column are significantly different according to Fisher's least significant difference test ($\alpha = 0.05$).

Table 4. Comparison of the effects of metam-sodium (Divapan) on microsclerotia viability reductions (%) when applied to different *Calonectria* spp. in plastic containers under virtually impermeable film (VIF) or totally impermeable film (TIF) in autumn 2014 (experiment I)

	Reductions (%) per rate^z							
	250 liters/ha		350 liters/ha		400 liters/ha		700 liters/ha	
Isolate	**VIF**	**TIF**	**VIF**	**TIF**	**VIF**	**TIF**	**VIF**	**TIF**
Calonectria brassicae LPF290	100 a	75.3 abc	100 a	79 bc	100 a	100 a	100 a	100 a
C. hodgesii LPF244	76.6 b	100 a	98.8 ab	100 a	100 a	100 a	100 a	100 a
C. hodgesii LPF245	58 bcd	51.9 b–f	100 a	81.5 ab	100 a	100 a	100 a	100 a
C. colombiana LPF075	58 bc	42 c–g	69.1 c–f	84 ab	100 a	100 a	100 a	100 a
C. brassicae LPF034	48.2 cde	66.7 a–e	100 a	92.6 ab	100 a	100 a	100 a	100 a
C. pteridis LPF004	44.4 c–f	46.9 b–f	55.6 fg	100 a	100 a	100 a	100 a	100 a
C. zuluensis LPF219	44.4 cde	43.2 b–f	77.8 a–f	100 a	100 a	100 a	100 a	100 a
C. spathulata LPF066	43.2 c–f	28.4 f–i	33.3 ghi	70.4 bc	100 a	100 a	100 a	100 a
C. pini LPF253	42 cde	35.8 e–h	59.3 efg	70.4 bcd	100 a	100 a	100 a	100 a
C. sulawesiensis LPF389	35.8 c–f	18.5 f–i	93.8 abc	76.6 bc	100 a	91.4 b	100 a	100 a
C. pini LPF031	34.6 d–g	34.6 e–h	67.9 def	100 a	100 a	100 a	100 a	100 a
C. orientalis LPF300	32.1 def	55.6 b–f	84 a–d	84 ab	100 a	100 a	100 a	100 a
C. ovata LPF257	27.2 e–h	70.4 a–d	54.3 efg	100 a	100 a	100 a	100 a	100 a
C. pseudoscoparia LPF061	25.9 e–i	33.3 e–i	80.3 a–e	40.7 e	100 a	100 a	100 a	100 a
C. orientalis LPF301	24.7 e–h	50.6 b–f	86.4 a–d	58 cde	100 a	100 a	100 a	100 a
C. ovata LPF174	23.5 f–i	77.8 ab	75.2 a–d	100 a	100 a	100 a	100 a	100 a
C. brachiatica LPF130	21 e–i	81.5 abc	100 a	100 a	100 a	100 a	100 a	100 a
C. variabilis LPF220	9.9 ghi	100 a	30.9 gh	100 a	100 a	100 a	100 a	100 a
C. variabilis LPF007	3.7 hi	66.7 a–e	72.8 a–f	70.4 bc	100 a	100 a	100 a	100 a
C. pseudoscoparia LPF212	0 i	56.8 b–f	77.8 a–f	69.1 bcd	100 a	100 a	100 a	100 a
C. brasiliensis LPF388	0 i	51.9 b–f	69.1 b–f	50.6 de	100 a	100 a	100 a	100 a
C. ecuadoriae LPF452	0 i	42 c–h	100 a	92.6 ab	100 a	100 a	100 a	100 a
C. brachiatica LPF195	0 i	40.7 deh	14.8 hi	53.1 cde	100 a	100 a	100 a	100 a
C. pteridis LPF002	0 i	7.4 ghi	64.2 def	33.3 ef	100 a	100 a	100 a	100 a
C. naviculata LPF311	0 i	7.4 ghi	18.5 hi	11.1 f	100 a	100 a	100 a	100 a
C. hongkongensis LPF111	0 i	0 i	4.9 i	11.1 f	61.7 b	13.6 c	93.8 b	96.3 b

^{z} Each value represents the mean of three replicates, each consisting of 54 infected carnation pieces. Recovery values followed by different letters within each column are significantly different according to Fisher's least significant difference test ($\alpha = 0.05$).

Table 5. Comparison of the effects of dazomet (Basamid) on microsclerotia viability reductions (%) when applied to different *Calonectria* spp. in plastic containers under virtually impermeable film (VIF) or totally impermeable film (TIF) in spring 2015 (experiment II)

	Reductions (%) per rate[z]					
	100 kg/ha		**160 kg/ha**		**200 kg/ha**	
Isolate	**VIF**	**TIF**	**VIF**	**TIF**	**VIF**	**TIF**
Calonectria brachiatica LPF130	100 a	100 a	100 a	100 a	100 a	100 a
C. pteridis LPF004	100 a	100 a	100 a	100 a	100 a	100 a
C. ovata LPF174	100 a	100 a	100 a	100 a	100 a	100 a
C. zuluensis LPF219	100 a	100 a	100 a	100 a	100 a	100 a
C. colombiana LPF075	100 a	100 a	100 a	100 a	100 a	100 a
C. pseudoscoparia LPF212	100 a	100 a	100 a	100 a	100 a	100 a
C. variabilis LPF007	95.1 ab	100 a	100 a	100 a	100 a	100 a
C. hodgesii LPF245	90.1 abc	100 a	100 a	100 a	100 a	100 a
C. hodgesii LPF244	87.7 abc	88.9 ab	100 a	100 a	100 a	100 a
C. ovata LPF257	86.4 abc	19.8 ab	100 a	100 a	100 a	100 a
C. brassicae LPF290	84 abc	100 a	100 a	100 a	100 a	100 a
C. ecuadoriae LPF452	72.8 bcd	100 a	100 a	100 a	100 a	100 a
C. brassicae LPF034	71.6 cd	77.8 ab	100 a	100 a	100 a	100 a
C. spathulata LPF066	66.7 cd	100 a	100 a	100 a	100 a	100 a
C. pseudoscoparia LPF061	66.7 cd	66.7 bc	100 a	100 a	100 a	100 a
C. brachiatica LPF195	58 de	100 a	100 a	100 a	100 a	100 a
C. pini LPF031	49.4 de	61.7 bc	100 a	100 a	100 a	100 a
C. pini LPF253	48.2 de	49.4 cd	100 a	100 a	100 a	100 a
C. brasiliensis LPF388	35.8 ef	33.3 de	100 a	100 a	100 a	100 a
C. pteridis LPF002	34.6 ef	100 a	100 a	100 a	100 a	100 a
C. sulawesiensis LPF389	33.3 ef	66.7 bc	100 a	100 a	100 a	100 a
C. hongkongensis LPF111	21 f	32.1 de	33.3 c	33.3 c	66.7 b	66.7 b
C. naviculata LPF311	16 f	11.1 e	33.3 c	46.9 b	56.8 b	100 a
C. orientalis LPF300	11.1 f	69.1 bc	100 a	100 a	100 a	100 a
C. orientalis LPF301	11.1 f	48.2 cd	100 a	100 a	100 a	100 a
C. variabilis LPF220	7.4 f	100 a	76.5 b	100 a	100 a	100 a

[z] Each value represents the mean of three replicates, each consisting of 54 infected carnation pieces. Recovery values followed by different letters within each column are significantly different according to Fisher's least significant difference test ($\alpha = 0.05$).

Table 6. Comparison of the effects of metam-sodium (Divapan) on microsclerotia viability reductions (%) when applied to different *Calonectria* spp. in plastic containers under virtually impermeable film (VIF) or totally impermeable film (TIF) in spring 2015 (experiment II)

	Reductions (%) per rate[z]			
	250 liters/ha		**350 liters/ha**	
Isolate	**VIF**	**TIF**	**VIF**	**TIF**
Calonectria brassicae LPF290	100 a	79.2 abc	100 a	83.3 c
C. pseudoscoparia LPF212	80.3 abc	100 a	100 a	100 a
C. sulawesiensis LPF389	75.3 a–d	70.4 cde	100 a	90.1 b
C. hodgesii LPF244	72.7 ab	100 a	100 a	100 a
C. orientalis LPF300	70.4 b–e	80.3 bcd	100 a	100 a
C. spathulata LPF066	69.2 b–e	85.2 abc	65.4 d–g	100 a
C. ovata LPF257	66.7 b–f	100 a	100 a	100 a
C. colombiana LPF075	64.2 b–f	70.4 cde	100 a	100 a
C. naviculata LPF311	63 b–f	33.3 fgh	86.4 bcd	58 d
C. hodgesii LPF245	55.6 b–g	86.4 abc	100 a	100 a
C. pini LPF253	51.9 c–g	63 c–f	100 a	100 a
C. ovata LPF174	50.6 c–g	85.2 abc	58.1 efg	100 a
C. pteridis LPF004	49.4 e–h	84 abc	92.6 abc	100 a
C. brachiatica LPF130	49.4 c–g	63 c–f	100 a	100 a
C. brachiatica LPF195	45.7 c–g	95.1 ab	40.7 efg	100 a
C. zuluensis LPF219	44.5 e–h	88.9 ab	100 a	100 a
C. brassicae LPF034	44.4 d–h	100 a	100 a	100 a
C. brasiliensis LPF388	42 e–h	54.3 d–g	86.4 abc	98.8 ab
C. orientalis LPF301	42 e–h	44.5 e–h	87.7 abc	100 a
C. variabilis LPF220	35.8 fgh	100 a	74.1 c–f	100 a
C. variabilis LPF007	34.6 gh	98.8 ab	88.9 abc	100 a
C. pini LPF031	33.3 gh	81.5 abc	51.9 fg	100 a
C. ecuadoriae LPF452	32.1 fgh	48.1 efg	100 a	100 a
C. hongkongensis LPF111	32.1 gh	30.9 h	51.9 g	38.3 e
C. pseudoscoparia LPF061	22.2 hi	100 a	97.5 ab	100 a
C. pteridis LPF002	0 i	33.3 gh	79 cde	100 a

[z] Each value represents the mean of three replicates, each consisting of 54 infected carnation pieces. Recovery values followed by different letters within each column are significantly different according to Fisher's least significant difference test ($\alpha = 0.05$).

experiment. The efficacy of the fumigants and rates on *C. polizzii* ITEM 14877 and *C. pauciramosa* ITEM 14884 microsclerotia viability and the relative VIF and TIF performances at each fumigant rate in experiments III, IV, and V in the soil under open-field conditions were also examined using an analysis of variance. The mean separation of the viability reduction percentages compared with relative controls in plastic containers among isolates, the fumigant effects on *C. polizzii* and *C. pauciramosa* survival rates in soil under open-field conditions, and the relative infectivity levels (disease incidence) in red clover were assessed using Fisher's least significance difference test at $\alpha = 0.05$.

Results

Effects of DZ and MS on exotic *Calonectria* microsclerotia. Isolate, rate, film, and their interactions always significantly affected the number of viable microsclerotia buried in the fumigated plots (Table 2). TIF significantly enhanced the performance of fumigation when compared with VIF over independent experiments (Table 2). Data from experiments I and II are reported in Tables 3, 4, 5, and 6).

Because all of the interactions were significant (Table 2), the responses to fumigation among the isolates were analyzed and shown for single fumigation rates under the same film (Tables 3, 4, 5, and 6).

In experiment I, Basamid (DZ) at 400 and 500 kg/ha resulted in the elimination of microsclerotia viability for all of the *Calonectria* spp. whereas, at 200 kg/ha, viability was eliminated in all of the species, except *C. naviculata*. When this fumigant was applied at 160 kg/ha, no viable microsclerotia were retrieved from fumigated containers for any species, except for *C. variabilis* LPF220, *C. hongkongensis*, and *C. naviculata* isolates. This fumigant was less effective when applied at 100 kg/ha, resulting in the elimination of microsclerotia viability for 16 of 26 and 21 of 26 isolates under VIF and TIF, respectively. The highest variability of response to DZ fumigation was detected at 100 kg/ha of the commercial formulate (Basamid; Table 3). Divapan (MS) at 1,000 liters/ha eliminated the microsclerotia viability of all species or isolates whereas, at 700 and 400 liters/ha, viable inocula were recovered only from *C. sulawesiensis* and *C. hongkongensis*. The commercial formulate (Divapan) at 250 and 350 liters/ha showed weaker capabilities to reduce the microsclerotia viability of *Calonectria* spp. or isolates, and these concentrations resulted in the greatest variability in response to fumigation among the tested isolates (Table 4).

In experiment II, the highest rates of Basamid (DZ) showed excellent efficacies at 400 and 500 kg/ha in killing the microsclerotia of all *Calonectria* spp. This fumigant applied at 160 and 200 kg/ha totally reduced the microsclerotia viability of all species, except for *C. naviculata, C. variabilis* LPF220, and *C. hongkongensis*, whereas, at 100 kg/ha, it was, on average, less effective compared with the data of experiment I. At this fumigant rate, the elimination of microsclerotia viability was detected for only 6 of 26 and 14 of 26 isolates under VIF and TIF, respectively (Table 5).

Divapan (MS) at 400, 700, and 1,000 liters/ha eliminated *Calonectria* microsclerotia viability, whereas the 350 liters/ha rate killed the most *Calonectria* isolates under TIF (21 of 26) and half of the isolates under VIF. The commercial formulate applied at 250 liters/ha was the least efficient in reducing the viability of *Calonectria* inocula. As in the previous experiment, a high rate of reduction in viability among *Calonectria* isolates was detected at the low fumigation rates (Table 6).

Effects of reduced fumigant rates on the survival of *C. polizzii* and *C. pauciramosa* microsclerotia. Data from experiments III, IV, and V, performed in open fields, are reported in Table 7. In experiment III, all of the treatments killed microsclerotia of *C. polizzii*

Table 7. Effectiveness of reduced rates of dazomet (Basamid) and metam-sodium (Divapan) applied to loamy-sand soil in affecting survival (%) of *Calonectria polizzii* and *C. pauciramosa* from infected carnation leaves after exposure to fumigation treatment under virtually impermeable film (VIF) or totally impermeable film (TIF) soil mulchings in the nursery in three experiments[y]

Treatment, rate^z	Experiment III (summer 2013)				Experiment IV (autumn 2013)				Experiment V (spring 2014)			
	C. polizzii		*C. pauciramosa*		*C. polizzii*		*C. pauciramosa*		*C. polizzii*		*C. pauciramosa*	
	VIF	TIF	VIF	TIF	VIF	TIF	VIF	TIF	VIF	TIF	VIF	TIF
Divapan (400)	0	0	0	0	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
Divapan (350)	0	0	0	0	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
Divapan (250)	0	0	0	0	9.9 b*	0 a*	24.7 b*	0 a*	0 a	0 a	0 a	0 a
Basamid (200)	0	0	0	0	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
Basamid (160)	0	0	0	0	0 a	0 a	0 a	0 a	0 a*	19.1 b*	0 a	0 a
Basamid (100)	0	0	0	0	13 b^{ns}	17.9 b^{ns}	36.4 c*	9.3 b*	1.2 a*	20.4 b*	18.5 b^{ns}	16.7 b^{ns}
Control	100	100	100	100	100 c	100 c	100 d	100 c	100 b	100 c	100 c	100 c

[y] Each value represents the mean of three replicates, each consisting of 54 infected carnation pieces. Recovery values followed by different letters within each column or by an asterisk (*) within each row are significantly different according to Fisher's least significant difference test ($\alpha = 0.05$); ns = not significant.

[z] Rates: liters/hectare for Divapan or kilograms/hectare for Basamid.

Table 8. Disease incidence (%) of crown and root rot caused by *Calonectria pauciramosa* and *C. polizzii* on red clover seedlings grown in peat substrate mixed with carnation leaf pieces colonized by pathogen microsclerotia retrieved from both fumigated and untreated plots[y]

Treatment, rate^z	Infectivity of *C. polizzii*						Infectivity of *C. pauciramosa*					
	Experiment III		Experiment IV		Experiment V		Experiment III		Experiment IV		Experiment V	
	VIF	TIF	VIF	TIF	VIF	TIF	VIF	TIF	VIF	TIF	VIF	TIF
Divapan (400)	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a
Divapan (350)	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a
Divapan (250)	0.0	0.0	15.8 b*	0.0 a*	0.0 a	0.0 a	0.0	0.0	28.6 b*	0.0 a*	0.0 a	0.0 a
Basamid (200)	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a
Basamid (160)	0.0	0.0	0.0 a	0.0 a	0.0 a*	28.1 b*	0.0	0.0	0.0 a	0.0 a	0.0 a	0.0 a
Basamid (100)	0.0	0.0	17.2 b	20.5 b	4.2 a*	27.6 b*	0.0	0.0	40.5 c*	13.6 b*	21.2 b	23.4 b
Control	88.5	89.6	90.3 c	85.7 c	93.8 b	89.2 c	90.3	87.4	88.4 d	84.6 c	92.8 c	87.2 c

[y] Data presented are means of three replications (each consisting of 42 to 55 young red clover seedlings). Arcsine ($\sin^{-1}$ square root x) transformation was used on percentage data prior to analysis; untransformed data are presented. VIF = virtually impermeable film and TIF = totally impermeable film. Disease incidence values followed by different letters within each column or by * within each row are significantly different according to Fisher's least significant difference test ($\alpha = 0.05$). Infectivity levels of fumigated pathogen microsclerotia are always compared with those of relative controls.

[z] Rates: liters/hectare for Divapan or kilograms/hectare for Basamid.

and *C. pauciramosa* independent of fumigant, rate, and film. In experiment IV, all of the treatments were effective in eliminating the inocula of *C. polizzii* and *C. pauciramosa*, except the lowest rates of Basamid (DZ) (under both films) and Divapan (MS) (under VIF), which significantly reduced the viable inocula of each species compared with control. At these rates, TIF increased the efficacy of fumigant treatments compared with VIF. In the last experiment (V), only Basamid (DZ) failed to kill all of the viable microsclerotia of these two *Calonectria* spp. when applied at 100 kg/ha and of *C. polizzii* at 160 kg/ha under TIF. Significant differences related to fumigant rate and film were observed for *Calonectria* spp. survival but TIF did not improve the efficacies of treatments when compared with VIF.

Evaluation of the viability of *C. polizzii* and *C. pauciramosa* microsclerotia on red clover following fumigation treatments. Microsclerotia infectivity rates on young red clover seedlings in experiments III, IV, and V are reported in Table 8. In experiment III, no *Calonectria* infections were observed on preinfected carnation debris after retrieval from the fumigated plots. This corroborated the pathogen survival rate of zero. The disease incidence was strongly correlated to the survival of *Calonectria* microsclerotia. Thus, in experiment IV, *Calonectria* infections were only found on infected debris recovered from plots treated with Basamid (DZ) under VIF or TIF at a rate of 100 kg/ha and Divapan (MS) under VIF at 250 liters/ha. *Calonectria* infections on red clover were only obtained from microsclerotia exposed to fumigation at a rate of 160 kg/ha under TIF and at 100 kg/ha under both films. The pairwise combinations of the significant differences between treatments were similar to those observed for the survival experiments.

Discussion

This article provides initial information on the efficacies of DZ and MS fumigants applied at reduced rates under low-permeability films under nursery conditions against 19 *Calonectria* spp., representative of a worldwide population.

In the greenhouse experiments, both DZ and MS were effective in reducing exotic *Calonectria* microsclerotia viability, although some differences in their performances were observed between experiments. Both Basamid (DZ) and Divapan (MS), when applied at 160 kg/ha and 400 liters/ha concentration and greater, respectively, under either film were effective in eradicating *Calonectria* microsclerotia in soil, except for *C. hongkongensis* (viable with Divapan at up to 700 liters/ha and Basamid up to 200 kg/ha), *C. sulawesiensis* (viable with Divapan at up to 400 liters/ha), and *C. variabilis* LPF220 and *C. naviculata* (viable with Basamid at up to 160 and 200 kg/ha, respectively). At lower rates, there was greater variability in microsclerotia variability among most *Calonectria* isolates in all of the experiments. In general, TIF enhanced fumigant performance. Because high inter- and intraspecific variability of responses to fumigants was detected among *Calonectria* spp. or isolates, the correct identification of target *Calonectria* spp. could play an important role in the choice of fumigant and application rate.

The field data confirmed the efficacy of the highest rates of both fumigants against *C. polizzii* and *C. pauciramosa*, as similarly reported by Polizzi et al. (2014). In comparison with a previous study that utilized higher labeled rates (Divapan and Basamid at 400, 700, and 1,000 liters/ha and 200, 400, and 500 kg/ha, respectively), the reduced rates (defined as less than the labeled rate) applied under VIF and TIF were also effective in reducing both endemic *Calonectria* spp. in soil. In addition, the efficacy of the fumigant treatment appeared to be strongly influenced by the season and, to a lesser extent, by the film used. Indeed, no viable inocula were retrieved from any of the fumigated plots in the July experiment. In the remaining experiments (in November and May), the lowest rates of fumigants were less effective and did not eliminate viable inocula of either pathogen. Moreover, TIF did not always improve the efficacy of the treatment when compared with VIF. The highest *Calonectria* infection rates were obtained from inocula retrieved from untreated plots and, to a lesser extent, from plots treated at the lowest fumigant rates.

The current labeled rates are 300 liters/ha in open fields and 700 to 1,200 liters/ha in greenhouses for Divapan 51 while the rates for Basamid range from 300 to 500 kg/ha (at a 15-to 20-cm depth) for some pathogens to 500 to 700 kg/ha (at a 25- to 30-cm depth) for other pathogens. Our data clearly showed the possibility of reducing the application rates by up to 160 kg/ha for Basamid (DZ) and 400 liters/ha for Divapan (MS) against all *Calonectria* inocula in soil or substrate used for ornamental and forestry crops when using low-permeability films (VIF or TIF). High rates of MS should be applied only for *C. hongkongensis, C. sulawesiensis, C. variabilis*, and *C. naviculata.* The use of Basamid (DZ) applied at 100 kg/ha and Divapan (MS) at 250 liters/ha in July could be encouraged for the eradication of endemic *C. polizzii* and *C. pauciramosa* under nursery conditions in the Mediterranean Basin. DZ and MS have been previously reported as effective against other soilborne pathogen inocula but at higher rates than those reported in this article (Wang et al. 2006; Weiland et al. 2011, 2013).

TIF is less permeable than VIF, and it allows a decrease in fumigant application rates by 40 to 50% without sacrificing fumigant efficacy (Fennimore and Ajwa 2011; Gao et al. 2013; McAvoy and Freeman 2013). However, the fumigant efficacy in our study was not always significantly greater under TIF compared with VIF. Nevertheless, TIF could be particularly attractive because it decrease gas emissions. High-permeability films (low-density and high-density polyethylene) should be replaced by low-permeability films to reduce or kill *Calonectria* inocula as well as already was reported for other soilborne fungal pathogens (Cabrera et al. 2015; Chamorro et al. 2016) because they are more environmentally friendly (Gao et al. 2011) and allow for the use of lower fumigant rates.

This investigation indicated that fumigation at reduced rates under low-permeability films is an appropriate option to reduce or kill soilborne *Calonectria* spp. before growing seedlings, propagating plants, or replanting pot-grown plants in nurseries. Because the most widely used potting substrate is derived from mixture of local (volcanic) soil with commercial peat and mineral constituents, the sustainable disinfection of soil or substrate should be encouraged on a large scale as an additional agronomic practice in nursery production.

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